Presence of Vascular Endothelial Growth Factor in the Growth Plate of Chickens with Rickets and Tibial Dyschondroplasia

Sarah Easter
Spring 2003
Faculty and Project Advisor: Dr. Latshaw

Acknowledgement

The Ohio State Department of Animal Sciences and Dr. David Latshaw are enthusiastically acknowledged for supporting the execution of this project.

Abstract

The goal of this experiment was to test for the presence of Vascular Endothelial Growth Factor (VEGF) in the growth plates of young chickens with rickets and tibial dyschondroplasia (TD). Normal growth plates were also harvested for comparison. The normal growth plate occurred with 1% calcium in the diet. Rickets was induced in two ways. A calcium deficiency was created by feeding 0.6% calcium in the diet, and a phosphorus deficiency was created by feeding .25% phosphorus in the diet. TD was induced by feeding 0.8% calcium, which is considered an intermediate calcium deficiency. The growth plates were harvested when the chicks were 19 days old, and were tested for the presence of VEGF. Other tissue samples were taken for testing against the anti-VEGF antibody in order to assure that the antibody being used would react with poultry VEGF. Portions of the breast, liver, and spleen were all harvested. The tissues were stored in a paraformaldehyde solution and were taken to the histology lab at The Ohio State College of Veterinary Medicine. The tissues were then embedded in paraffin and sectioned. After sectioning, the paraffin was removed and the tissues were re-hydrated. Immunohistochemistry was performed to test for the growth factor by using an anti-VEFG antibody and a secondary marker that indicated the places the anti-VEGF antibody

reacted within the tissue. A human anti-VEFG antibody produced in rabbits was used because there was no commercially available anti-VEGF antibody specifically for poultry. VEGF is reportedly highly conserved among species, so a reaction between the human antibody and the chicken growth factor was highly probable. Unfortunately, no reaction was detected in the poultry tissues while testing with the human anti-VEFG antibody.

Introduction

A normal growth plate in the proximal tibia of a chicken exhibits 3 zones (Figure 1). The zones are the zone of proliferation, the zone of prehypertrophy, and the zone of hypertrophy. The area above these zones consists of articular cartilage. The cells within the zone of proliferation are flat and elongated, and the zone is vascularized. The zone of prehypertrophy contains cells that are small and round. This zone is not normally vascularized. The third zone is the zone of hypertrophy. This zone contains larger round cells, and is vascularized.

Rickets is a condition of insufficient mineralization of the cartilage at the growing ends of the bone (Shore and Pozanski, 1996). Rickets is recognizable by the increase in size and disorganized appearance of the zone of proliferation within the growth plate. In chickens with rickets, the zone of proliferation and the zone of prehypertrophy are vascularized. This condition can be caused in poultry by restricting the dietary calcium, phosphorus, and/or vitamin D (Toure et al., 2002). When rickets is caused by a

phosphorus deficiency, the growth plate appears to have more vascularization than when rickets is induced by a calcium deficiency.

Tibial Dyschondroplasia (TD) is also an abnormality in the growth plate. In TD, the growth plate is not calcified or vascularized, and it appears enlarged (Praul et al., 2000). The cartilage within the growth plate increases in size and does not follow the normal progression to become ossified. TD can be caused in the laboratory setting by feeding diets only slightly deficient in calcium (Toure et al., 2002). TD can also be the result of the genetic makeup of the bird. Commercial broiler crosses have been bred to improve otherwise common leg problems, such as TD (Kestin et al., 1999).

Vascular Endothelial Growth Factor (VEFG) is a growth factor that signals for vascularization of the cartilaginous material within the growth plate. This means that VEGF allows blood vessels to penetrate the cartilage within the growth plate. Vascularization of the cartilage is necessary to allow for the deposition of minerals and the creation of bone because it provides access for the necessary cells to enter the region (Lin et al., 2002). These cells will break down the cartilage and deposit minerals to create bone growth. Without this vascularization, the growth plates will not ossify properly. The lack of VEGF in the growth plate is a probable cause for the abnormal vascularization in the growth plate of chickens with rickets, and the nonexistent vascularization within the growth plate of chickens with TD. Praul et al. (2000) determined that a condition similar to TD could be caused by blocking VEGF signaling in the growth plate of chickens. This research provides more evidence for the idea that the absence of VEGF in the growth

plate of chickens is a major determining factor in the onset of TD, and possibly for the case of rickets as well.

Hypotheses

- The growth plates from chickens with calcium-induced tibial dyschondroplasia will show little or no VEGF expression.
- The growth plates from chickens with calcium-induced rickets will show more VEGF expression than the growth plates from chickens with tibial dyschondroplasia. The growth plates from chickens with phosphorus-induced rickets will show more VEGF expression than either calcium-induced TD or calcium-induced rickets due to increased vascularization within the growth plate.

Materials and Methods

For this study, four groups of chickens were raised: normal, calcium-induced rickets, phosphorus-induced rickets, and calcium-induced TD. The normal chickens were raised as the control group. These chicks were fed 1.0% calcium in their diets. To produce chicks with TD, a mild calcium deficiency was induced by feeding a diet of 0.8% calcium. Calcium-induced rickets was caused in the chicks by feeding a diet of 0.6% calcium, and phosphorus-induced rickets was caused by feeding a diet containing 0.25% phosphorus.

The chicks were grown to 19 days of age and were reared in battery brooders. Five chicks from each group were chosen at random for collection of the growth plates.

Two of the five chicks were randomly chosen within each group for the collection of additional tissues. The additional tissues included parts of the liver, spleen, and breast muscle. These tissues provided a control for testing with the anti-VEGF antibody. The tissues were stored in a cooled paraformaldehyde solution. This is the same as 10% neutral buffered formalin, which is commonly used by veterinarians. Slide preparation and further testing were performed at the histology lab at The Ohio State University College of Veterinary Medicine. Five slides were made for each sample.

The normal procedures for dehydration, sectioning, and performing an immunohistochemistry are as follows. The tissues are fixed with a paraformaldehyde solution. Being "fixed" means that the cells are held in place for testing. The tissues are then dehydrated with various alcohol solutions, starting with 50% ethanol three times for twenty minutes each time. Next, the tissues are placed in a 70% ethanol three times for twenty minutes each time. They could be stored in the solution of 70% ethanol for a few days if necessary. In order to complete the dehydration process, the samples are placed in a 95% ethanol solution three times for twenty minutes each time, then in a 100% ethanol solution three times for twenty minutes each time. Finally, the 100% ethanol solution is replaced by xylene, which is added three times for ten minutes each time. These steps are all performed at room temperature. After the tissues are dehydrated, they are embedded in paraffin in order to precisely cut the tissue. This step, also referred to as sectioning, is extremely important and sensitive. It is necessary to have this step performed by a well-trained individual so the tissue is not ruined in the process. The tissues can be stored in the paraffin stage for years if they are kept dry. After the tissues are cut properly, the paraffin

is removed from the tissue (Ausubel, 1996). The tissues are then rehydrated so immunohistochemistry can be performed. Upstate Cell Signaling Solutions (2003) suggests deparaffinizing by placing the sample in xylene three times for five minutes each time, then rehydrating by placing the tissues in 100% ethanol twice for five minutes each time, in 95% ethanol twice for five minutes each time; and once in 80% ethanol for five minutes. After this, the antibody is placed on the slide for the time recommended for the specific antibody. The first antibody will not be visible, so a secondary marker must be applied to produce a colored region where expression of the protein occurs (Ausubel, 1996).

Since VEGF is an intracellular protein, an additional step was needed to allow the anti-VEGF antibody to react with the VEGF protein at its full potential. This step is intended to disrupt the cell membranes, and occurs between rehydration and addition of the antibody to the slides. The histology lab used two different methods to achieve this goal. First, the slides were placed in steam. No reaction occurred with this method, so a second method was attempted. The enzyme, trypsin, was added to the slides. Trypsin is used to achieve the same ultimate goal, but is more abrasive to the cells. No reaction was detected using this method either, indicating that this step probably did not the cause of the lack of reaction.

To perform immunohistochemistry for this experiment, an anti-VEGF antibody that would label the areas where VEGF was being expressed was chosen. There was no commercially available avian anti-VEGF antibody, but it was known that the genetic makeup of VEGF is highly conserved among the species. Taking this information one step

further, a search for genetically similar VEGF protein was performed. This was important in helping to choose an anti-VEGF antibody that would have a high probability of reacting with the chicken VEGF. This search was performed on the National Center for Biotechnology Information (NCBI) website. Using the results of this search (Figure 2), a human anti-VEGF antibody produced in rabbits was chosen for use in immunohistochemistry performed on the chicken growth plates.

The antibody used for this experiment was purchased from Oncogene Research Products[1], catalog #PC37. According to the company, the antibody was generated by immunizing rabbits with part of the human VEGF protein. Because human VEGF was similar to chicken VEGF, the human anti-VEGF was chosen for use in the immunohistochemistry reaction. Immunohistochemistry was performed on all of the growth plates and other tissue samples taken from the chickens. However, no reaction was detected on any of the samples.

Results

Rickets, TD, and normal growth plates were successfully induced in the previously mentioned manner. Figure 3 shows the gross appearance of some of the growth plates. These growth plates show the progression in appearance from a normal growth plate to cases where calcium is lacking in the diet. It is not easy to distinguish the areas within the normal growth plate in this picture. The growth plate in the middle of the picture shows

[1] 10394 Pacific Center Court
San Diego, California 92121 USA

signs of calcium rickets. This is evident in the increased zone of proliferation and the somewhat unorganized differentiations between the zones within the growth plate. It can be identified as a calcium deficiency because the zones do not contain an abundance of vessels like that of phosphorus rickets. The growth plate shown on the far right is a case of TD. This is extremely apparent due to the enlarged cartilaginous area. This area is easy to identify because it has no blood vessels.

Figure 4 shows the appearance of growth plates from increasingly deficient amounts of phosphorus in the diet. The growth plate on the left is a normal growth plate. The zones within this growth plate are easy to identify and are normal in size. There are also distinct separations between the zones within the normal growth plate. The phosphorus deficient growth plates exhibit rickets much like the calcium deficient growth plates do, but the area appears to contain more vessels and less white areas. The zones are also more irregular than in the normal growth plate. The middle growth plate is an even higher phosphorus deficiency. The zones are not distinguishable in this case, and the entire growth plate appears vascularized.

The anti-VEGF antibody used in this experiment did not react with any of the chicken tissues tested. A positive reaction for the presence of VEGF was expected in the liver, spleen, and breast tissues since they are vascularized tissues, but no reaction was detected. Although the human VEGF is genetically similar to the chicken VEGF, it is likely that there were enough differences to prevent the human anti-VEGF antibody from reacting with the chicken VEGF. The only conclusion that can be drawn at this time is that

the human anti-VEGF antibody used in this experiment will not react with chicken VEGF and should be avoided in future experiments with chicken VEGF detection.

Discussion

If a reaction had been detected in the tissues, the growth plates could have been analyzed in a qualitative manner. A system for comparison of the levels of VEGF expression in the different slides could have been set up. The growth plates could also have been analyzed quantitatively with the assistance of laboratory equipment. Only at that time could significant conclusions about the presence of VEGF in the growth plates of chickens with rickets and TD be drawn.

If VEGF does not appear to be the determining factor in the onset of TD and rickets, there are other possibilities that may be tested. There are many steps in the angiogenesis pathway, of which VEGF is just one part. Chemoattractants for vascular endothelial cells and matrix metalloproteinases are also necessary for vascularization of the growth plate (Lin et al., 2002). If these factors are not present, vascularization will not occur. All of these things are needed together, in the proper order, to create the complete pathway for angiogenesis and vascularization.

Because multiple slides were made for each tissue, it will be possible to perform this experiment again without utilizing more chickens. The slides can be stored for a long period of time because they are paraffinized. This project could be repeated using an anti-VEGF antibody from another species, or by using an avian anti-VEGF when it is created.

VEGF has many functions in the growth of an animal. It is involved in the production of most tissues because it is needed to allow vessels to grow in a specific area. The effect of VEGF on an animal is positive most of the time, but can also be negative. When VEGF is produced in large quantities, it can lead to tumor growth, and often, cancer.

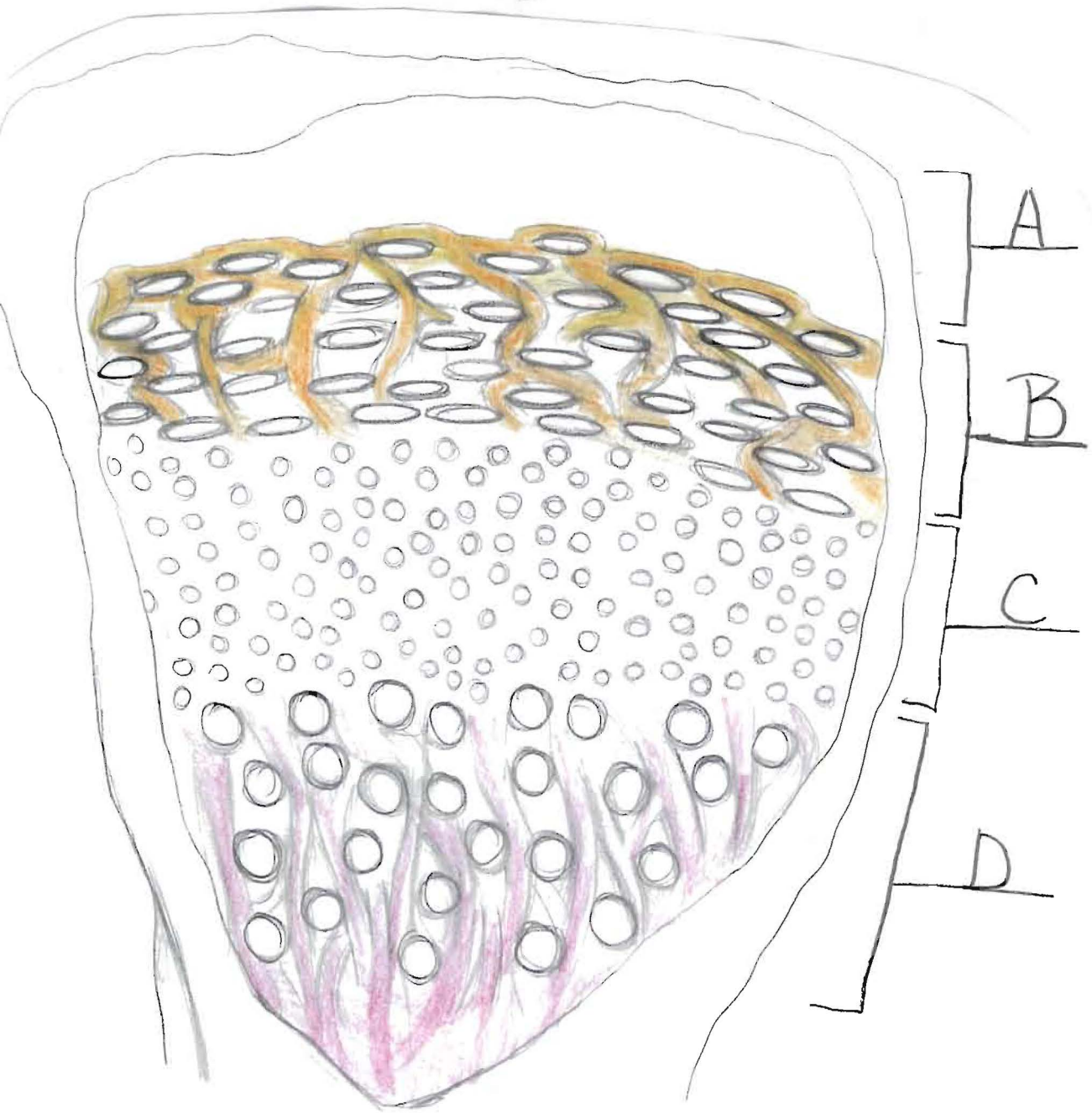
VEGF has been extensively studied in the growth of animal and human tumor cells for cancer research. VEGF seems to be the determining factor in tumor growth for most types of human cancers, including ovarian, colon, kidney, bladder, and lung cancers (Faviana, 2002). In many cases, if VEGF can be blocked, the tumor cannot continue to grow. Jayson et al. (2002) showed that malignant tumors could be reduced in size by subjecting them to an anti-VEGF antibody. The antibody was originally produced by mice and was changed in the laboratory into a human antibody. There was a decrease in tumor size in all of the patients within 48 hours. The decrease varied considerably from patient to patient.

There have also been studies conducted to find the factors that allow VEGF to be expressed in an uncontrolled manner, allowing for tumor growth. In human colon cancer, a mutated p53 gene is linked to the over-expression of VEGF. The p53 gene is a tumor suppressor gene. In a normal state, p53 does just that; it suppresses the growth of tumor cells. One study showed a strong correlation between the p53 gene and the expression of VEGF. It seems that when the p53 gene is mutated, VEGF is expressed in large amounts, and tumor growth in the colon is the result (Faviana, 2002).

Conclusion

Little research has been conducted specifically on the expression of VEGF within the proximal tibia of chickens. One hypothesis for this project is that the growth plates from chickens with calcium-induced TD would naturally show little or no VEGF expression. More research is needed to test this hypothesis, and the implications of this research reach far beyond the boundaries of poultry science. If it is found to be true that chickens can produce a naturally occurring block for the expression of VEGF, it could have an important relationship with cancer research. The results from this research would be beneficial in understanding the role of VEGF in poultry growth. In addition, it has the potential to lead to exciting, new approaches in the battle against malignant tumors.

Figure 1

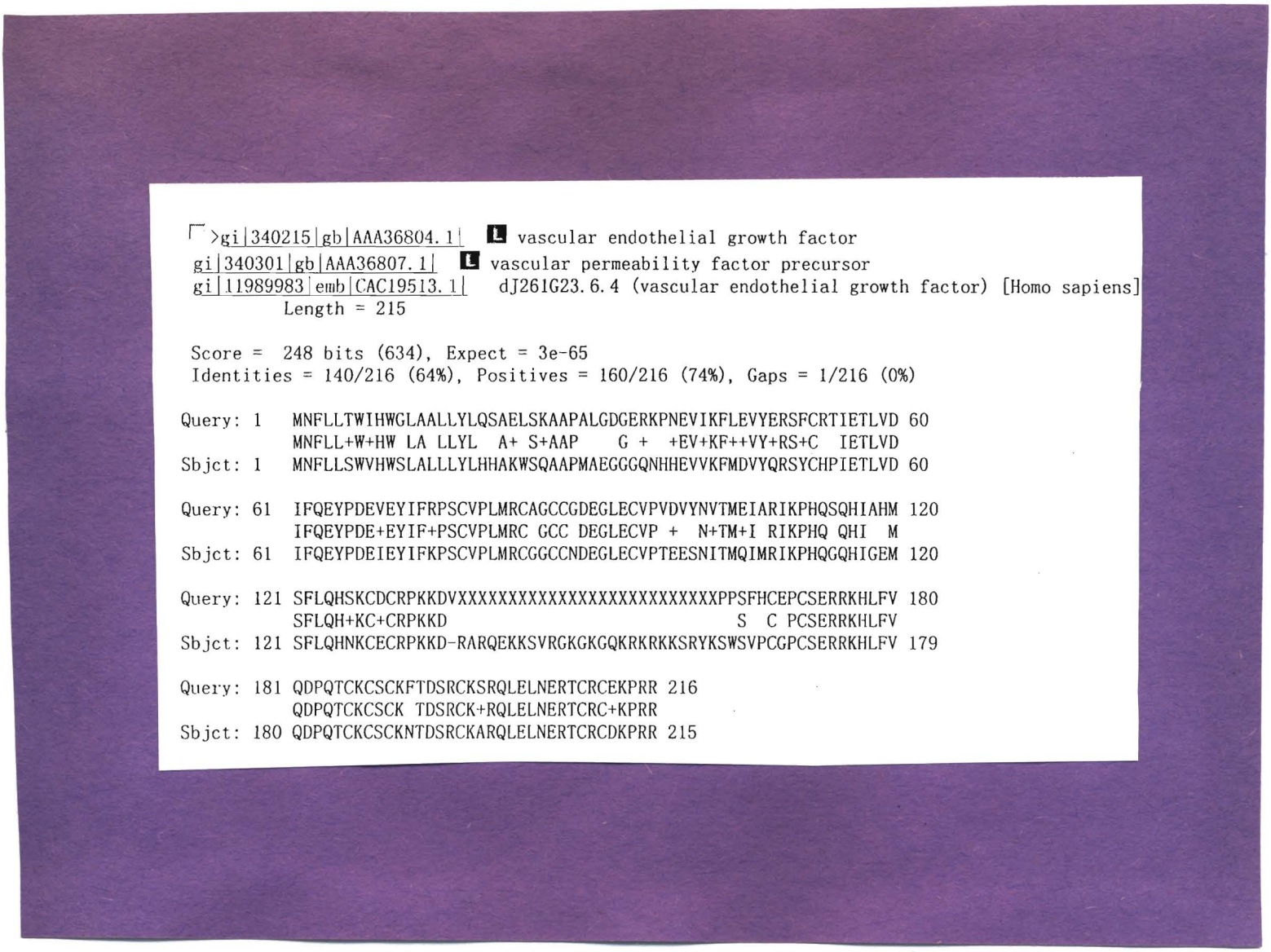


Microscopic view of the growth plate within the proximal tibia

A. Articular Cartilage
B. Zone of Proliferation
C. Zone of Prehypertrophy
D. Zone of Hypertrophy

Drawing by: Adam Chizmar

Figure 2



```
>gi|340215|gb|AAA36804.1|  vascular endothelial growth factor
gi|340301|gb|AAA36807.1|  vascular permeability factor precursor
gi|11989983|emb|CAC19513.1|  dJ261G23.6.4 (vascular endothelial growth factor) [Homo sapiens]
          Length = 215

 Score =  248 bits (634), Expect = 3e-65
 Identities = 140/216 (64%), Positives = 160/216 (74%), Gaps = 1/216 (0%)

Query: 1   MNFLLTWIHWGLAALLYLQSAELSKAAPALGDGERKPNEVIKFLEVYERSFCRTIETLVD 60
           MNFLL+W+HW LA LLYL  A+ S+AAP    G +  +EV+KF++VY+RS+C  IETLVD
Sbjct: 1   MNFLLSWVHWSLALLLYLHHAKWSQAAPMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVD 60

Query: 61  IFQEYPDEVEYIFRPSCVPLMRCAGCCGDEGLECVPVDVYNVTMEIARIKPHQSQHIAHM 120
           IFQEYPDE+EYIF+PSCVPLMRC GCC DEGLECVP +  N+TM+I RIKPHQ QHI  M
Sbjct: 61  IFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTEESNITMQIMRIKPHQGQHIGEM 120

Query: 121 SFLQHSKCDCRPKKDVXXXXXXXXXXXXXXXXXXXXXXXXXXPPSFHCEPCSERRKHLFV 180
           SFLQH+KC+CRPKKD                                S  C PCSERRKHLFV
Sbjct: 121 SFLQHNKCECRPKKD-RARQEKKSVRGKGKGQKRKRKKSRYKSWSVPCGPCSERRKHLFV 179

Query: 181 QDPQTCKCSCKFTDSRCKSRQLELNERTCRCEKPRR 216
           QDPQTCKCSCK TDSRCK+RQLELNERTCRC+KPRR
Sbjct: 180 QDPQTCKCSCKNTDSRCKARQLELNERTCRCDKPRR 215
```

Comparison of human VEGF and poultry VEGF as provided by the National Center for Biotechnology website: www.ncbi.nlm.nih.gov/blast/Blast.cgi

Figure 3

From left to right: normal growth plate, calcium induced rickets, calcium induced TD

Figure 4

From left to right: normal growth plate, rickets induced by a moderate phosphorus deficiency, rickets induced by a severe phosphorus deficiency

Bibliography

Faviana, P., L. Boldrini, R. Spisni, P. Berti, D. Galleri, R. Biondi, T. Camacci, G. Materazzi, R. Pingitore, P. Miccoli, and G. Fontanini, 2002. "Neoangiogenesis in colon cancer: Correlation between vascular density, vascular endothelial growth factor (VEGF) and p53 protein expression." *Oncology Reports* 9: 617-620.

Jayson, Gordon C.; Jamal Zweit; Alan Jackson; Clive Mulatero; Peter Julyan; Malcolm Ranson; Lynn Broughton; John Wagstaff; Leif Hakannson; Gerard Groenewegen; John Bailey; Nigel Smith; David Hastings; Jeremy Lawrance; Hamied Haroon; Tim Ward; Alan T. McGown; Meina Tang; Dan Levitt; Sandrine Marreaud; Frederic F Lehmann; Manfred Herold; Heinz Zwierzina, 2002. "Molecular imaging and biological evaluation of HuMV833 anti-VEGF antibody: implications for trial design of antiangiogenic antibodies." *Journal of the National Cancer Institute*. 94 (19): 1484-1493.

Kestin, S.C.; G. Su; P. Sorensen, 1999. "Different Commercial Broiler Crosses Have Different Susceptibilities to Leg Weakness." *Poultry Science* 78: 1085-1090.

Lin, R.; N. Amizuka, T. Sasaki, M. M. Aarts, H. Ozawa, D. Goltzman, J. E. Henderson, and J. H. White, 2002. "1 alpha, 25-Dihydroxyvitamin D3 Promotes Vascularization of the Chondro-Osseus Junction by Stimulating Expression of Vascular Endothelial Growth Factor and Matrix Metalloproteinase 9." *Journal of Bone and Mineral Research* 17: 1604-1611.

Praul, C.A.; B.C. Ford, C.V. Gay, M. Pines, and R.M. Leach, 2000. "Gene Expression in Tibial Dyschondroplasia." *Poultry Science* 79: 1009-1013.

Shore, R. M., and A. K. Pozanski,1996. "Radiologic Evaluation of Bone Mineral in Children." *Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism*. Favus, Murray J., ed. Philadelphia: Lippincott-Raven Publishers.

Toure, I.; S. Weisbrode, and D. Latshaw, 2002. "Mild to Moderate Calcium Deficiency and Inclusion of Ammonium Chloride Affect the Onset of Tibial Dyschondroplasia and Rickets." *Journal of Animal and Veterinary Advances* 1: 149-154.

Upstate Group, Inc., 2003. "Immunohistochemistry with Fixed Paraffin-Embedded Tissue Sections." www.upstate.com/misc/protocols.q.prot.e.ihcprotocol/ Immunohistochemistry +with+Fixed+Paraffin+Embedded+Tissue+Sections.

Watkins, Simon, 1996. "In situ Hybridization and Immunohistochemistry." *Current Protocols in Molecular Biology.* Ausubel, F. M., ed. (14): 0.3-0.4; 6.1-6.13, John Wiley & Sons, Inc.